

### AMENDMENTS TO THE SPECIFICATION

Please amend the second full paragraph on page 6 to the second paragraph of page 7 as the follows:

For example, the following mutations are postulated to result in decreased metal binding affinity:

X<sub>1</sub>    [[=>]] is any one of A, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y;

X<sub>2</sub>    [[=>]] is any one of A, F, G, I, K, L, P, Q, R, S, T, V, W, Y;

X<sub>3</sub>    [[=>]] is any one of A, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y;

X<sub>4</sub>    [[=>]] is any one of A, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y,

and mutation of a side-chain to introduce a metal binding ligand that is likely to give rise to increased or modulated metal affinity include:

X<sub>5</sub>    [[=>]] is any one of C, D, E, H (this is already a His residue in pig albumin);

X<sub>6</sub>    [[=>]] is any one of C, D, E, H;

X<sub>7</sub>    [[=>]] is any one of C, D, E, H;

X<sub>2</sub>    [[=>]] is any one of C, D, E, H;

X<sub>4</sub>    [[=>]] is any one of C, E, H;

X<sub>1</sub>    [[=>]] is any one of C, D, E;

X<sub>3</sub>    [[=>]] is any one of C, D, E.

The inventors have found that metal binding at the proposed site is influenced by fatty acid binding (A. J. Stewart, C. A. Blindauer, S. Berezenko, D. Sleep, P. J. Sadler, *Proc. Natl. Acad. Sci. USA* 100, 3701-3706 (2003)[[.]] ). Comparison of the X-ray structures of fatty acid free albumin and albumin with 5 molecules of myristate (pdb 1bj5) bound reveal that, in order to accommodate a fatty acid anion in the so-called binding site 2, the long helix connecting domains I and II bends, and the two half-sites in unliganded rHA move by more than 10 Å to form a continuous cavity (Curry, S., Mandelkow, H., Brick, P. & Franks, N. (1998) *Nat. Struct. Biol.* 5,827-835[[.]]). This fatty acid binding results in a movement of residues H247 and D249 by 4-6 Å away from the other two residues, H67 and N99, in the proposed Zn site (see Figure X3c (a & b). D249 also changes its side-chain conformation to maintain the H-bond to N' of H67 and forms an additional H bond to N99. H247, which is H-bonded to N99 in the unliganded structure, forms an H bond with E100 in the fatty acid-

bound structures. The proposed switching of the zinc site in human albumin by fatty acid binding is an intriguing example of an allosteric interaction between an organic nutrient and an essential metal ion. Since the H247-E100 H-bond is expected to stabilise the "switched" form, it is predicted that the following mutations of E100 might influence the interactive metal/fatty acid binding[.]:

X8    [[=>]] is any one of A, C, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y;

Additionally, more recent studies have revealed that albumins with the mutations H67A, N99D, and N99H display properties dramatically different from the wild-type when used in cell culture media. Cell adhesion is impaired in both the H67A and N99H mutants. It is known that uptake by the liver of, e. g., fatty acids from albumin involves non-specific binding of albumin to the cell surface, and an induced conformational change of the albumin molecule (R. G. Reed, C. M. Burrington, J. Biol. Chem. 264, 9867-9872, 1989). The mutated residues are all involved in stabilising domain I-domain-II contacts via H.bonds. The finding that a single mutation at the domain I/II interface has a severe impact on the effect of the mutated albumin on cells suggests that the following mutations, which refer to domain I/II histidine residues involved in inter-domain H bonds, can also have similar impact on cell adhesion and/or growth[.]:

X9    [[=>]] is any one of A, D, E, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y;

X10   [[=>]] is any one of A, D, E, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y;

X11   [[=>]] is any one of A, D, E, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y;

Please replace the section titled "Detailed description of the invention" which runs from page 14 to page 16 as the follows:

#### **Detailed description Description of the Invention**

The present invention will now be further described by way of example and with reference to the figures, which show:

Figure 1 shows a model of the three dimensional structure of human serum albumin as reported in PDB 1AO6, with the metal binding site identified herein, highlighted shown in the boxed area;

Figure 2 shows in more detail amino acid side-chains located in and around the proposed zinc binding site;

Figure 3a shows an initial model of zinc site in wild-type albumin, in comparison with apo-rHA (1A06).

Figure 3b shows recalculated, improved model of a zinc site in wild-type human serum albumin, in comparison with apo-rHA (1A06). Force-field energy of the zinc site: 59.1 kcal/mol;

Figure 3c shows model for the metal site in the Asn99His mutant, in comparison with wild-type Zn rHA (~~green~~). Force field energy for the zinc site is 83.2 kcal/mol. rmsd to wild-type apo: 0.54 Å; to wild-type Zn-albumin: 0.56 Å;

Figure 3d shows model for the metal site in the Asn99Asp mutant;

Figure 3e shows inter-domain H bonds at the potential zinc site in models of zinc-free wild-type and mutant albumins. a: Wild-type; b: Fatty-acid loaded wild-type; c: Asn99His mutant model; d: Asn99Asp mutant model;

Figure 4 shows circular dichroism spectra of wild type (solid line), and H67A (dashed line) albumin;

Figure 5 shows  $^{111}\text{Cd}$  NMR of native and H67A rHA with 2 mol equivalent of  $^{111}\text{CdCl}_2$ ;

Figure 6 shows  $^{111}\text{Cd}$  NMR of rHA with 2 mol equivalent of  $^{111}\text{CdCl}_2$  in the presence of a) zinc and b) copper;

Figure 7 shows UV-visible absorption spectra of (a) native rHA and (b) H67A rHA with 0.2 to 2 mol equivalent of  $\text{CuCl}_2$  in 0.2 mol equivalent steps (bottom to top);

Figure 8 shows the potential zinc binding site in an ~~asn99asp~~ Asn99Asp mutant without zinc bound. ~~shown in magenta~~ Shown on the right side overlay is the wild-type structure. ~~the~~ The force-field energy of the mutated site (101.4 kcal/mol) is insignificantly higher than that of the wild-type (55.6 kcal/mol) and the ~~asn99his~~ Asp99His site (75.6 kcal/mol).

Figure 9 shows the 1D  $^{111}\text{Cd}$  NMR spectra of recombinant albumins (wild-type and Asn99His mutant) with 2 mol equivalents of  $^{111}\text{Cd}^{2+}$  (conditions: 1 mM protein, 50 mM Tris-Cl, 50 mM NaCl, 295 K);

Figure 10 shows the 1D  $^{111}\text{Cd}$  NMR spectra of recombinant albumins (wild-type and Asn99Asp mutant) with 2 mol equivalents of  $^{111}\text{Cd}^{2+}$  (conditions: 1 mM protein, 50 mM Tris-Cl, 50 mM NaCl, 295 K if not stated otherwise);

Figure 11 shows the titrations of 1 mM rHA with copper (II) (pH 7.4, 0.2 M potassium phosphate).  $\text{CuCl}_2$  was added in 0.2 mol equiv portions in each case. Shown are difference spectra, corrected for the absorption of albumin;

Figure 12 shows the direct comparison of the effect various amounts of  $\text{Cu}^{2+}$  on the UV-Vis difference spectra of wild-type and mutant albumin.

Figure 13 shows deconvoluted FT-ICR-MS spectrum of wild-type rHA (20  $\mu\text{M}$  in 8 mM  $\text{NH}_4\text{Ac}$ , 25% methanol, 1% acetic acid). Note the narrow line shape (half-height width ca. 25 Da) which enables the detection of small-molecule adducts;

Figure 14a shows a survey of resolution-enhanced 1D  $^1\text{H}$  NMR spectra of recombinant albumin mutants. ~~Figures 14b, c, d, and e show portions of 2D TOCSY NMR spectra of wild-type, His67Ala, Asn99His, and Asn99Asp rHA, respectively, showing His H $\delta$ 2/H $\epsilon$ 1 cross-peaks.~~ All samples were 1 mM in 50 mM Tris-Cl, 50 mM NaCl, and all experiments were carried out at 310 K. pH values vary between 7.28 (N99H) and 7.40 (H67A), which accounts for slight differences in chemical shifts for individual protons. Observable H $\epsilon$ 1 protons are labelled with numbers, f denotes formate, which had been added as a chemical shift standard;

Figure 15 shows portions of 1D (a) and 2D TOCSY spectra (b) with 1 mol equiv  $\text{Zn}^{2+}$  added (pH\* = 7.37) showing histidine H $\delta$ 2/H $\epsilon$ 1 cross-peaks;

Figure 16a shows portions of resolution enhanced 1D NMR spectra of wild-type rHA (1 mM in 50 mM Tris-Cl, 50 mM NaCl, pH\* = 7.26) with varying amounts of octanoate; Figure 16b shows the effect of increasing amounts of octanoate on chemical shifts of histidine H $\epsilon$ 1 protons;

Figures 17a & b show titration of  $\text{Cd}_2\text{rHA}$  with octanoate. Conditions: 1 mM rHA, 2 mol equiv  $\text{CdCl}_2$ , 50 mM Tris-Cl, 50 mM NaCl, 10%  $\text{D}_2\text{O}$ , pH 7.1, 298 K, 10 mm BBO probe. The acquisition of one spectrum typically takes 4 hours. The graph in 17b shows the time-course for 4 equivalents [As the acquisition of each spectrum takes 4 h, the mid-point

(i.e. two hours after starting the experiment) of each spectrum has been taken as the average time-point];

Figure 18a shows cell counts in layer using native and mutant serum albumins;

Figure 18b shows percentage of dead cells in layer using native and mutant serum albumins;

Figure 18c shows cell counts in medium using native and mutant serum albumins; and

Figure 18d shows percentage of dead cells in medium using native and mutant serum albumins.

Figure 19 shows mutation identified as being involved domain I-domain II contacts via H bonds.

Figures 20a and 20b show Table 1, a comparison of amino acid sequence between mammalian albumins. Residues, in the locus of the zinc binding site are highlighted. Amino acids before the N terminal amino acid (residue number 1), in the boxed area, are part of the pre-albumin sequence and are cleaved following translation to give albumin itself. Accession numbers of the sequences are Human, P02768; Macaque, M90463; Canine, CAB64867; Feline, P49064; Bovine, P02769; Sheep, P14639; Pig, ABPGS; Rabbit, P49065 and Rat, P02770.

Please delete pages 35 and 36. The Table 1 contained therein is made into new Figures 20a and 20b in the Drawings. The figure legend is moved to the end of the section titled "Detailed Description of the Invention."